H^+/Ca^{2+} exchange driven by the plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana* reconstituted in proteoliposomes after calmodulin-affinity purification

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Abstract The plasma membrane Ca^{2+}-ATPase was purified from *Arabidopsis thaliana* cultured cells by calmodulin (CaM)-affinity chromatography and reconstituted in proteoliposomes by the freeze-thaw sonication procedure. The reconstituted enzyme catalyzed CaM-stimulated $^{45}Ca^{2+}$ accumulation and H^+ ejection, monitored by the increase of fluorescence of the pH probe pyranine entrapped in the liposomal lumen during reconstitution. Proton ejection was immediately reversed by the protonophore FCCP, indicating that it is not electrically coupled to Ca^{2+} uptake, but it is a primary event linked to Ca^{2+} uptake in the form of countertransport.

Key words: Ca^{2+}-ATPase; Calmodulin; H^+/Ca^{2+} antiport; Plasma membrane; Proteoliposome; Pyranine; *Arabidopsis thaliana*

1. Introduction

Calcium extrusion from the cytoplasm of plant cells to the apoplast plays an important role in the long-term maintenance of the steep Ca^{2+} gradient across the plasma membrane (PM), as well as in reestablishing the low basal cytosolic Ca^{2+} concentration after a stimulus-induced Ca^{2+} entry through PM channels. Moreover, it is likely involved in determining the shape and amplitude of Ca^{2+} waves which are thought to confer specificity to Ca^{2+} signaling [1–3].

The PM of plant cells is endowed with a calmodulin (CaM)-regulated Ca^{2+}-ATPase belonging to the same subfamily (type IIB) of P-type ATPases as its animal counterpart [4–7]. Like the PM Ca^{2+}-ATPase of animal cells [8,9], the plant PM Ca^{2+}-ATPase binds CaM at an autoinhibitory domain which can be selectively cleaved by controlled trypsin treatment [10–12]. However, the plant PM Ca^{2+}-ATPase has some distinctive biochemical and structural features which clearly differentiate it from the PM Ca^{2+}-ATPase of animal cells. Among the biochemical features it is worth recalling the low specificity for nucleoside triphosphates, unique to plant type IIB Ca^{2+}-ATPases, which are the only Ca^{2+}-transporting P-type ATPases able to use GTP or ITP nearly as well as ATP [7,13–16]. Recently, cloning of the first plant PM Ca^{2+}-ATPase gene (*At*-ACA8, [4]) has revealed a major structural difference from animal PM Ca^{2+}-ATPase in the location of the CaM binding domain. In contrast to the animal enzyme, which has an extended C-terminal domain which contains the CaM binding domain [8,9], the plant PM Ca^{2+}-ATPase has a very short cytosolic C-terminus and an extended N-terminus which contains the CaM binding domain [4]. This characteristic makes the plant PM Ca^{2+}-ATPase similar to type IIB Ca^{2+}-ATPases localized on endomembranes of plant cells [7,15].

The biochemical characteristics of the Ca^{2+}-ATPase in native PM vesicles have been described in some detail [7,15,17], but little is known about its transport mechanism. The PM Ca^{2+}-ATPase of animal cells has been shown to catalyze an H^+/Ca^{2+} exchange [8,18–20]. Some pieces of evidence coming from both biochemical and physiological studies indicate that also the PM Ca^{2+}-ATPase of higher plant works as an H^+/Ca^{2+} exchanger [3,21]. However, a thorough investigation of the problem in vivo or in native PM vesicles is hampered by the presence of the much more abundant H^+-ATPase, which pumps H^+ in the opposite direction [22], and possibly by the activity of a secondary H^+/Ca^{2+} antiport, which has been found in PM from maize leaves [23].

We have set the procedure for the purification of the plant PM Ca^{2+}-ATPase by CaM-affinity chromatography [4,24] and have exploited it to address the problem of the mechanism of Ca^{2+} transport in reconstituted proteoliposomes. The obtained results show that the PM Ca^{2+}-ATPase purified from *Arabidopsis thaliana* cultured cells does indeed catalyze an H^+/Ca^{2+} exchange.

2. Materials and methods

2.1. Plant material and isolation of PM vesicles

PM was purified from cell suspension cultures of *A. thaliana* (L.) ecotype Landsberg by aqueous two-phase partitioning as previously described [4,11].

2.2. CaM-affinity purification of PM Ca^{2+}-ATPase and reconstitution in proteoliposomes

PM proteins were solubilized with *n*-dodecyl β-D-maltoside (1:1, mg detergent ml^{-1}:mg protein ml^{-1}) and purified by CaM-agarose (Sigma P4385)-affinity chromatography as described by Bonza et al. [4,24], with the exception that in the elution buffer the detergent polyoxyethylene-20-cetyl ether (Brij 58) was replaced with *n*-dodecyl β-D-maltoside (50 μg ml^{-1}), more suitable for the reconstitution procedure. The EDTA-eluted fraction, supplemented with equimolar

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Abbreviations: BTP, bis-tris propane (1,3-bis[tris (hydroxymethyl)-methylamino]-propane); CaM, calmodulin; EO, eosin Y; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PM, plasma membrane

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$CaCl_2$, was immediately dialyzed for 1 h at 4°C against a solution free of Ca^{2+} and EDTA and containing 30% (v/v) glycerol. After dialysis, one volume of 4 mg ml^{-1} L-α-phosphatidylcholine (type IV-S from soybean; Sigma P3644), dispersed by sonication under an N_2 stream in 4 mM bis-tris propane (BTP)–HEPES, pH 7, 200 mM KCl, 4 mM dithiothreitol, 10% (v/v) glycerol and 15% cholesterol (w/w, phosphatidylcholine/cholesterol), was mixed with three volumes of the dialyzed EDTA eluate (20:1, mg lipid ml^{-1}:mg protein ml^{-1}). The reconstitution was performed through two cycles of freeze-thaw sonication as described in Grandmougin-Ferjani et al. [25]. Finally, the proteoliposomes were passed through a Sephadex G100 exclusion column. When needed, pyranine (5 mM final concentration) was added prior to freeze-thaw sonication.

Protein was assayed according to Markwell et al. [26] after methanol precipitation [4].

Recovery of Ca^{2+}-ATPase activity in the reconstituted proteoliposomes was somewhat variable between preparations. Thus, the data reported in the tables and figures are from one experiment representative of at least two, performed on different proteoliposome preparations.

2.3. Electrophoresis and immunoblotting analysis

SDS–PAGE, silver staining, Western blotting and immunodecoration with anti-*At*-ACA8p [4], anti-*At*-ACA1p [27] and anti-PM H^+-ATPase [28] antisera were performed as previously described [4,24,28].

2.4. Measurements of the hydrolytic activity of the PM Ca^{2+}-ATPase

The hydrolytic activity of the PM Ca^{2+}-ATPase was measured as Ca^{2+}-dependent MgATP or MgITP hydrolysis [4,14]. The assay medium was the same as described by Bonza et al. [4]. The free Ca^{2+} concentration was buffered at 10 μM with 1 mM EGTA [29]. CaM (from bovine brain, Sigma P2277) was supplied at 20 μg ml^{-1}; incubation was performed at 25°C for 60 min. ATP or ITP hydrolysis measured in the presence of 1 mM EGTA without added Ca^{2+} was subtracted from the reported data. Assays were performed on three replicates and standard error of the assay did not exceed 3% of the reported values.

2.5. Ca^{2+} transport measurements

Ca^{2+} uptake was assayed by the membrane filtration technique as previously described [21]. Briefly, the proteoliposomes (ca. 1.5 μg of protein) were preincubated for 10 min at 20°C in 200 μl of assay medium containing 40 mM BTP–HEPES, pH 7, 3 mM $MgSO_4$, 50 mM KCl, 0.2 mM EGTA and $CaCl_2$ labeled with $^{45}Ca^{2+}$ (about 0.5 Bq $CaCl_2$ $pmol^{-1}$) to give a free Ca^{2+} concentration of 10 μM [29]. CaM was supplied at 10 μg ml^{-1}. The reaction was started by addition of 1 mM ATP or 1 mM ITP and stopped by dilution with 4 ml of 4 mM BTP–HEPES, pH 7, 5 mM $MgSO_4$, 50 mM KCl, 2 mM EGTA (blocking solution). The sample was then filtered under vacuum on an MF-Millipore (GSWP02500) membrane filter and the filter washed twice with 4 ml of blocking solution, allowed to dry and dissolved in 10 ml of Filter Count (Packard). Radioactivity was measured by liquid scintillation counting (Tri-carb LSC 1500, Packard). Assays were performed on three replicates and standard error of the assay did not exceed 5% of the reported values.

2.6. H^+ transport measurements

Lumenal H^+ ejection was measured by following the changes in the fluorescence intensity of the pH indicator pyranine entrapped in the proteoliposomes during the reconstitution procedure. Fluorescence intensity was measured using a λ_{exc} 450 nm and a λ_{em} 510 nm with a spectrofluorometer (Jasco FP-770). Proteoliposomes (ca. 2.5 μg protein), reconstituted in the presence of 5 mM pyranine, were incubated 5 min at 20°C in 1.8 ml of basal medium (40 mM BTP–HEPES, pH 7, 1 mM EGTA, 3 mM $MgSO_4$, 50 mM KCl) with additions specified in the legend to Fig. 3.

3. Results and discussion

3.1. CaM-affinity purification of PM Ca^{2+}-ATPase and reconstitution in proteoliposomes

In a first set of experiments we tried to reconstitute the Ca^{2+}-ATPase, purified from *A. thaliana* PM by CaM-affinity chromatography, by detergent dialysis. This procedure, which generates tight homogeneous proteoliposomes, has been successfully applied to reconstitute type IIB Ca^{2+}-ATPases purified from both the PM of animal cells and endomembranes of plant cells [30–33]. Unfortunately, the plant PM Ca^{2+}-ATPase did not survive the extensive dialysis required (data not shown), so we shifted to the more rapid freeze-thaw sonication procedure [25,30]. Since the recovery of PM Ca^{2+}-ATPase activity was very low when purification was performed in the absence of detergents (data not shown), purification was performed in the presence of *n*-dodecyl β-D-maltoside, which is well tolerated by the PM Ca^{2+}-ATPase [4,24] and forms relatively small micelles which can be easily removed by gel filtration. The gel filtration step also allowed removal from the extraliposomal medium of the pH probe pyranine, which was entrapped in the proteoliposomes during reconstitution [19,34]. Purification was performed as previously described [4,24], except that the EDTA-eluted fraction was immediately dialyzed against 30% (v/v) glycerol to reduce its volume and decrease the concentration of EDTA and Ca^{2+} prior to reconstitution.

Table 1
Purification and reconstitution of the PM Ca^{2+}-ATPase

Fraction	Total protein (mg)	PM Ca^{2+}-ATPase activity (nmol Pi min^{-1})	
		−CaM	+CaM
PM	4.5	185(41)	283(63)
Solubilized PM	4.2	168(40)	249(59)
CaM-agarose:			
Unbound	2.9	136(47)	145(50)
EDTA eluate	0.05	10(202)	41(814)
Proteoliposomes	0.04	7(175)	18(450)

PM proteins were solubilized, purified and dialyzed as described in Section 2. The reconstitution was performed through two cycles of freeze-thaw sonication followed by a passage through a Sephadex G100 exclusion column. The Ca^{2+}-ATPase activity reported is Ca^{2+}-dependent ITPase activity plus or minus 20 μg ml^{-1} CaM; ITPase activity measured in the absence of Ca^{2+} was (nmol Pi min^{-1}) 91 in the PM fraction, 85 in the solubilized PM, 90 in the fraction which did not bind to CaM-agarose and barely detectable in subsequent fractions (respectively 2.5 and 0.5 nmol Pi min^{-1} in the EDTA-eluted fraction and in the proteoliposomes). Data in parentheses represent the specific activities in nmol Pi min^{-1} mg^{-1} protein. Results are from one experiment, with three replicates, representative of 20.

The EDTA-eluted fraction so obtained contained about 15% of the loaded CaM-stimulated Ca^{2+}-ATPase activity (Table 1). In the assay conditions used (10 μM free Ca^{2+}), the percent stimulation of Ca^{2+}-ATPase activity by CaM ranged between 250 and 400%; this is likely an underestimation since CaM, beside increasing the V_{max} of the reaction, also decreases the apparent K_M of the enzyme for free Ca^{2+} [10,12,13], making the Ca^{2+}-ATPase more sensitive to inhibition by high Ca^{2+} concentrations (L. Luoni, unpublished results).

The EDTA-eluted fraction was highly enriched in a 123 kDa band (Fig. 1A, lane 1), which was identified as the PM Ca^{2+}-ATPase by immunodecoration with an antiserum against a portion of the N-terminus of the PM Ca^{2+}-ATPase (Fig. 1B, lane 1) absent in the CaM-regulated Ca^{2+}-ATPases of plant endomembranes [4]. In agreement with previous reports [4,12,24], the 123 kDa band (Fig. 1C, lane 1) also cross-reacted with an antiserum against the large cytosolic loop of

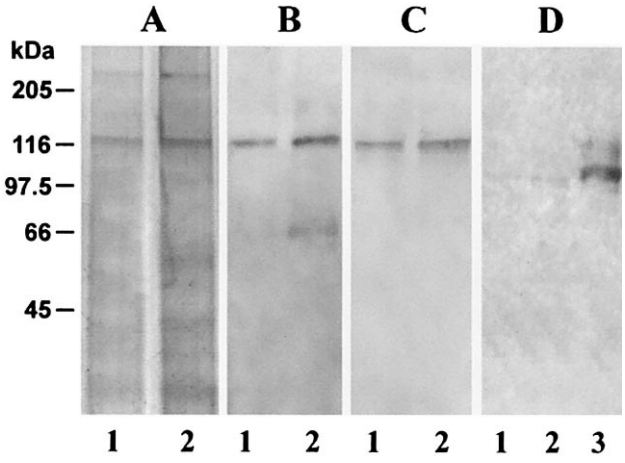

Fig. 1. Identification of PM Ca^{2+}-ATPase in the EDTA-eluted fraction (lanes 1) and in reconstituted proteoliposomes (lanes 2). The two fractions were separated by SDS–PAGE and stained with silver impregnation method (A) or blotted and immunodecorated with anti-*At*-ACA8 antiserum (B), anti-*At*-ACA1 antiserum (C) or with an antiserum against the PM H^+-ATPase (D). Lane 3 of panel D was loaded with native PM proteins. All lanes were loaded with the same Ca^{2+}-ATPase activity (ca. 0.08 nmol Pi min^{-1}). Numbers on the left indicate the size of molecular mass markers. Results are from one experiment representative of at least two.

At-ACA1p, a putative Ca^{2+}-ATPase of the plastid envelope [27]. Fig. 1D also shows that the EDTA-eluted fraction was virtually devoid of PM H^+-ATPase. In fact, an antiserum against the PM H^+-ATPase [28] hardly labeled any band in the EDTA-eluted fraction (lane 1), while it heavily labeled a 100 kDa band in lane 3, which was loaded with an amount of PM proteins corresponding to the same Ca^{2+}-ATPase activity loaded in lane 1.

After the reconstitution procedure, about two thirds of protein and half of the Ca^{2+}-ATPase activity were recovered in the proteoliposome fraction (Table 1). Stimulation by CaM (140–350%) was consistently lower in the proteoliposomes than in the EDTA-eluted fraction. This decrease might well be due to the presence of acidic phospholipids in the soybean phospholipids used for reconstitution; in fact, acidic phospholipids stimulate the PM Ca^{2+}-ATPase activity, mimicking to a large extent the effect of CaM (unpublished data from the authors' laboratory).

Comparison of signal intensities in lanes 1 (EDTA-eluted fraction) and 2 (proteoliposomes) of Fig. 1, which were loaded with equal Ca^{2+}-ATPase activity, indicates that the intensity of the 123 kDa band was higher (about 60%) in the lanes of proteoliposomes with all the detection methods applied. The faint band at about 70 kDa labeled by the antiserum against the N-terminus, visible in the proteoliposomes but not in the EDTA-eluted fraction (Fig. 1B), would suggest some proteolytic degradation of the Ca^{2+}-ATPase. However, it likely represents some cross-reacting contaminant introduced during the reconstitution procedure, since its intensity was very variable in different preparations and it was not labeled by the antiserum against the large cytosolic loop (Fig. 1C, lane 2) nor by iodinated CaM (not shown).

Altogether, these results indicate that the reconstitution procedure adopted caused a partial inactivation of the plant PM Ca^{2+}-ATPase, but allowed the recovery of a substantial amount of activity which was stable for at least 3 h (not shown).

3.2. Calcium transport driven by the reconstituted PM Ca^{2+}-ATPase

Fig. 2 shows the time course of Ca^{2+} uptake in proteoliposomes reconstituted with the PM Ca^{2+}-ATPase supplied with ATP, in the presence and in the absence of CaM. Supply of ATP initiated a substantial uptake of Ca^{2+} in the proteoliposomes which proceeded for at least 10 min: the uptake rate slowed down slightly already during the first minute and then progressively decreased. CaM strongly stimulated the uptake, its effect being maximal in the initial phase (about 200% in the experiment of Fig. 2). ATP-induced Ca^{2+} uptake was prevented by the Ca^{2+} ionophore A_{23187} (not shown), which induced the rapid release of Ca^{2+} taken up if supplied after uptake had proceeded for 5 min (Fig. 2).

Table 2 shows the main biochemical characteristics of Ca^{2+}-dependent nucleoside triphosphate hydrolysis and nucleoside triphosphate-dependent Ca^{2+} transport in the proteoliposomes reconstituted with the PM Ca^{2+}-ATPase. In agreement with what has been observed in native PM vesicles [14,29], the reconstituted PM Ca^{2+}-ATPase could use ITP as an alternative substrate, the hydrolysis rate being only slightly lower for ITP than for ATP. Similarly, Ca^{2+} uptake in the proteoliposomes was energized by ITP nearly equally as well as by ATP. Both Ca^{2+}-dependent ITP hydrolysis and ITP-dependent Ca^{2+} uptake were similarly stimulated by CaM and drastically inhibited by submicromolar concentrations of the fluorescein derivative eosin Y (EO, see [29]) and by vanadate.

Altogether these results clearly indicate that Ca^{2+} uptake in the proteoliposomes is driven by the PM Ca^{2+}-ATPase and

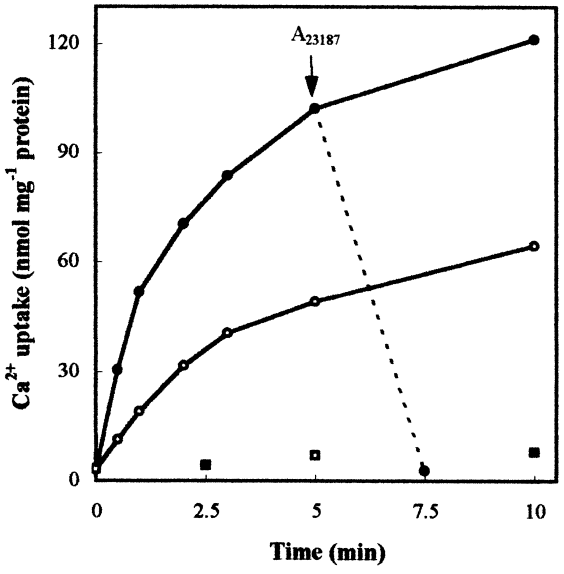

Fig. 2. Time course of ATP-dependent Ca^{2+} uptake in proteoliposomes reconstituted with the PM Ca^{2+}-ATPase, performed in the absence (○) and in the presence (●) of 20 μg ml^{-1} CaM. Proteoliposomes were preincubated for 10 min in assay medium before starting the reaction by addition of 1 mM ATP (time = 0). The arrow indicates addition of 5 μM A_{23187}. Ca^{2+} uptake in the absence of ATP was measured in the absence (□) and in the presence (■) of CaM. Results are from one of two experiments, each with three replicates.

Table 2
Effect of CaM and inhibitors on hydrolytic and Ca^{2+} transport activities of the reconstituted PM Ca^{2+}-ATPase

	Hydrolysis (nmol Pi min^{-1} mg $protein^{-1}$)	Ca^{2+} uptake (nmol Ca^{2+} min^{-1} mg $protein^{-1}$)
ATP	242	27
ATP+CaM	687	56
ITP	188	25
ITP+CaM	463	52
ITP+CaM+EO	7	5
ITP+CaM+vanadate	3	2

Assays were performed as described in Section 2. The hydrolytic activity reported represents the difference between activity measured in the presence of 10 μM free Ca^{2+} and in its absence. Ca^{2+} uptake was measured during the first minute after addition of nucleoside triphosphates; Ca^{2+} uptake measured in the absence of nucleoside triphosphates was subtracted from total Ca^{2+} uptake values. CaM, EO and vanadate were supplied at 20 μg ml^{-1}, 0.5 μM and 200 μM respectively. Results are from one experiment, with three replicates, representative of three.

that the purification–reconstitution procedure has no major effect on the biochemical characteristics of the enzyme.

The measured ratio between the initial rate of Ca^{2+} uptake and that of nucleoside triphosphate hydrolysis by the reconstituted plant PM Ca^{2+}-ATPase is about 0.1 (see Table 2). This low degree of coupling is probably due to the reconstitution procedure adopted: tight coupling between ATP hydrolysis and Ca^{2+} transport (1:1 ratio) by the PM Ca^{2+}-ATPase of animal cells could be obtained only when proteoliposomes were reconstituted by detergent dialysis [18] or by reverse phase evaporation [19,20]. Indeed, the finding that in our proteoliposomes the hydrolytic activity of the Ca^{2+}-ATPase is constant for at least 1 h also in the absence of the Ca^{2+} ionophore A_{23187} (not shown), while Ca^{2+} uptake levels off within the first 10 min (Fig. 2), suggests that the bulk of the Ca^{2+}-ATPase molecules is not involved in Ca^{2+} transport.

3.3. *Proton fluxes driven by the reconstituted PM Ca^{2+}-ATPase*

The fluorescent pH probe pyranine was entrapped in the proteoliposomes during the reconstitution procedure in order to measure changes in the intraliposomal pH caused by the activity of the PM Ca^{2+}-ATPase. Having a pK_a of 7.2 [34], pyranine is a suitable probe to measure any change in lumenal pH in proteoliposomes reconstituted in a pH 7 buffer. Moreover, being entrapped in the proteoliposomes its fluorescence is unaffected by H^+ production by the hydrolysis of nucleoside triphosphates.

Fig. 3A shows that supply of ITP to proteoliposomes incubated in the presence of Ca^{2+} and CaM initiated a time-dependent increase of pyranine fluorescence. This increase was absolutely dependent on the presence of Ca^{2+}, so that the reaction could be started alternatively by addition of ITP in the presence of Ca^{2+} or of Ca^{2+} in the presence of ITP. The signal was only slightly decreased in the presence of *p*-xylene-bispyridinium bromide, a water-soluble, non-permeant quencher of pyranine fluorescence [35] (not shown), indicating that it arose from intraliposomal pyranine and thus reported an alkalinization of the liposomal lumen.

Ca^{2+}-ITP-dependent intraliposomal alkalinization was strongly (ca. 200%) stimulated by CaM and drastically inhib-

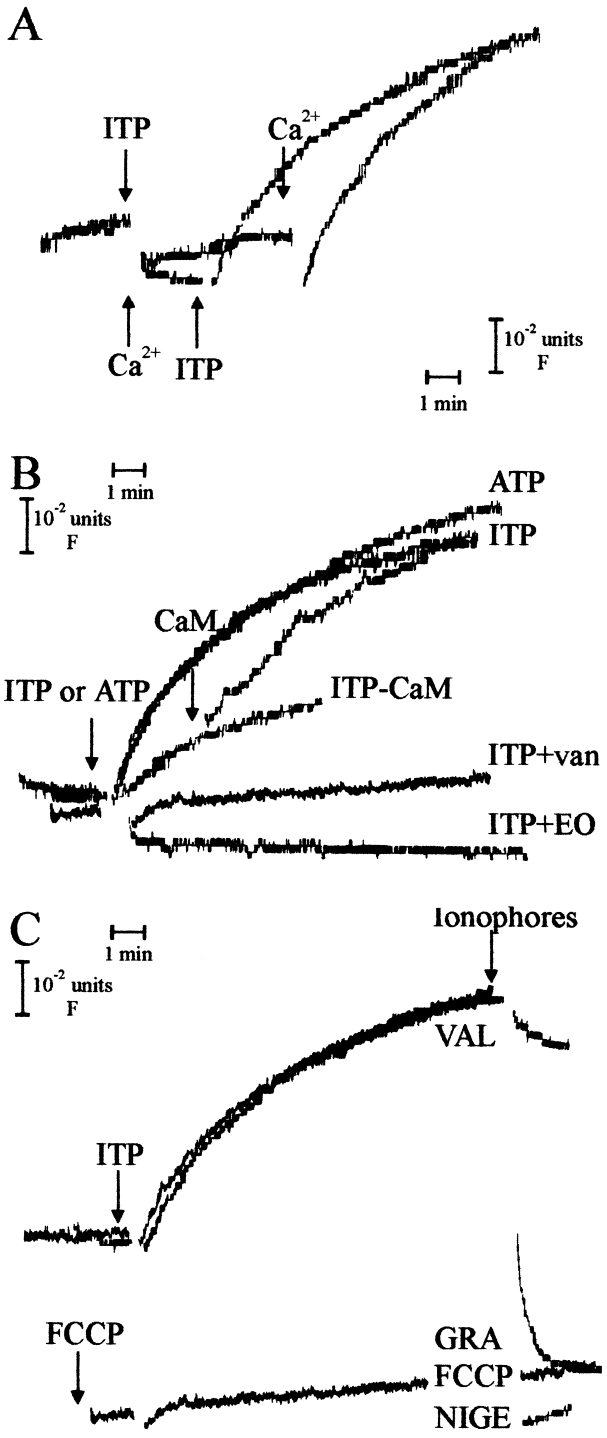

Fig. 3. Alkalinization of the intraliposomal pH by the reconstituted PM Ca^{2+}-ATPase. Proteoliposomes reconstituted in the presence of 5 mM pyranine were preincubated for 5 min at 20°C in basal assay medium (see Section 2) with the additions specified below. A: Preincubation was in basal medium supplemented with CaM. B: Samples were preincubated in the presence of Ca^{2+} and CaM (except for the −CaM trace, where CaM was added when indicated) plus or minus 0.5 μM EO or 200 μM vanadate. C: All samples were preincubated in the presence of Ca^{2+} and CaM. When specified by arrows, $CaCl_2$ was supplied to give a free Ca^{2+} concentration of 10 μM, CaM was supplied at 10 μg ml^{-1}, ITP or ATP at 1 mM, valinomycin (VAL) at 1 nM, nigericin (NIGE) and gramicidin (GRA) at 2 μM and FCCP at 5 μM. Results are from one of four experiments, each with two replicates.

ited by vanadate and EO (Fig. 3B). Moreover, it could be driven to a similar rate and extent by ITP or by ATP (Fig. 3B). Thus, it is concluded that proton ejection is driven by the reconstituted PM Ca^{2+}-ATPase.

Fig. 3C shows that CaM-stimulated Ca^{2+}-ITP-dependent intraliposomal alkalinization was nearly unaffected by the K^+ ionophore valinomycin, but immediately reversed by ionophores able to collapse proton gradients such as nigericin, gramicidin and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). The latter three ionophores decreased the pyranine fluorescence below the baseline measured before the addition of ITP: this results from an H^+ gradient across the proteoliposomal membrane, independent of the Ca^{2+}-ATPase activity. In fact, the same decrease was observed when FCCP was supplied before ITP (Fig. 3C); Ca^{2+}-ITP-dependent intraliposomal alkalinization was prevented under these conditions. Calcium uptake driven by the reconstituted PM Ca^{2+}-ATPase was unaffected by valinomycin and nigericin and slightly (20%) inhibited by FCCP, which similarly inhibited the hydrolytic activity of the enzyme [21].

The finding that proton ejection driven by the Ca^{2+}-ATPase is substantially insensitive to valinomycin, which under the experimental conditions used (equimolar K^+ in the medium and in the proteoliposomal lumen) collapses the transmembrane electrical potential difference, but prevented and completely reversed by FCCP, which specifically collapses the electrochemical H^+ gradient, indicates that it is not driven by a transmembrane electric potential difference generated by Ca^{2+} influx. Rather, H^+ ejection occurs against the electrochemical H^+ gradient and is the result of an H^+/Ca^{2+} exchange driven by the Ca^{2+}-ATPase.

4. Concluding remarks

The results reported in this paper represent the first direct demonstration that the PM Ca^{2+}-ATPase of plant cells catalyzes an H^+/Ca^{2+} exchange, and thus is able to use both the energy of hydrolysis of ATP and that of the electrochemical proton gradient built up by the H^+-ATPase to extrude Ca^{2+} from the cytoplasm to the apoplast. This characteristic has important physiological implications. In fact, plant cells maintain a concentration gradient of 10^4-fold across the PM, which, together with the transmembrane potential difference of ca. 180 mV, inside negative, makes a transmembrane electrochemical gradient for Ca^{2+} efflux of up to ca. 60 kJ mol^{-1} [1,2,22]. Thus, the energy of hydrolysis of ATP (about −46 kJ mol^{-1}) would not be enough to extrude Ca^{2+} across the PM under most physiological conditions. The extra energy supplied by H^+ influx according to the electrochemical H^+ gradient generated by the H^+-ATPase [22] would be essential to allow Ca^{2+} extrusion mediated by the PM Ca^{2+}-ATPase.

A stoichiometry of 1 H^+ per Ca^{2+} like that measured for the animal PM Ca^{2+}-ATPase [19,20] would be enough to allow Ca^{2+} extrusion from plant cells via the PM Ca^{2+}-ATPase under most physiological conditions, since H^+ influx can provide more than 30 kJ mol^{-1} (a pH gradient of two units is maintained by the PM H^+-ATPase even under basal conditions [22]). A $2H^+/Ca^{2+}$ stoichiometry has been estimated from H^+ and Ca^{2+} fluxes driven by the PM Ca^{2+}-ATPase in *Egeria densa* leaves [3]. We were unable to measure ITP-dependent changes in the transmembrane electrical potential difference in our reconstituted proteoliposomes. This result would be coherent with a $2H^+/Ca^{2+}$ stoichiometry, which would allow the PM Ca^{2+}-ATPase to work very far from its equilibrium under physiological conditions, but might also be due to intrinsic leakiness of the proteoliposomes. Hopefully, heterologous expression of the PM Ca^{2+}-ATPase cDNA [4] in yeast will soon provide a more abundant source of the enzyme [36], which will allow a more thorough investigation of the problem.

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